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Prototype development of the intelligent hydrogel wound dressing and its efficacy in the detection of model pathogenic wound biofilms

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ABSTRACT

The early detection of wound infection *in-situ* can dramatically improve patient care pathways and clinical outcomes. There is increasing evidence that within an infected wound the main bacterial mode of living is a biofilm: a confluent community of adherent bacteria encased in an extracellular polymeric matrix. Here we have reported the development of a prototype wound dressing, which switches on a fluorescent color when in contact with pathogenic wound biofilms. The dressing is made of a hydrated agarose film in which the fluorescent dye containing vesicles were mixed with agarose and dispersed within the hydrogel matrix. The static and dynamic models of wound biofilms, from clinical strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis*, were established on nano-porous polycarbonate membrane for 24, 48 and 72 hours, and the dressing response to the biofilms on the prototype dressing evaluated. The dressing indicated a clear fluorescent / color response within four hours, only observed when in contact with biofilms produced by a pathogenic strain. The sensitivity of the dressing to biofilms was dependent on the species and strain types of the bacterial pathogens involved, but a relatively higher response was observed in strains considered

good biofilm formers. There was a clear difference in the levels of dressing response, when dressings were tested on bacteria grown in biofilm or in planktonic cultures, suggesting that the level of expression of virulence factors is different depending of the growth mode. Colorimetric detection on wound biofilms of prevalent pathogens (*S. aureus*, *P. aeruginosa* and *E. faecalis*) is also demonstrated using an *ex-vivo* porcine skin model of burn wound infection.

KEYWORDS

Hydrogel dressing, Wound biofilm, Vesicles, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*

INTRODUCTION

When a wound is created it is soon colonized by bacteria from the surrounding skin and environmental sources, which include a range of opportunistic pathogens.^{1,2} In many cases, following initial contamination, the microbial population of wounds typically remains at a persistent low-level background colonization. This poses no significant impediment to wound healing and does not require treatment.³ In a sub-set of cases, the bacterial population does not stabilize at this sub-clinical level, but continues to expand to the point where the bacterial burden overwhelms the host immune clearance, allowing tissue invasion, and clinically relevant infection is initiated. This tipping point is often referred to as the critical colonization threshold (CCT), or simply critical colonization, and is generally regarded as the point at which clinical intervention becomes necessary.⁴ Despite the apparent importance of the CCT concept to pathogenesis of wound infection, its exact definition and tools to identify wounds approaching the CCT are yet to be developed.⁵

Many bacterial species also form encapsulated surface associated communities, termed biofilms, which pose particular problems in the treatment of wound infection.⁶⁻¹¹ These include the impairment of epithelialization and interference with formation of granulation tissues, while the reduced susceptibility of biofilms to host defenses and anti-microbial agents undermines standard clinical therapies, including antibiotic therapy.^{12,13} Biofilm formation is also considered a key factor in entry the wounds into a chronic non-healing state,³ and biofilms are estimated to play a role up to 60% of chronic wounds.¹⁴ Treatment costs associated with biofilm infection and chronic wounds may also be increased, and estimated to be more than 1 billion USD annually in

the United States,⁹ and to cost the UK NHS ~2.3-3.1 Billions GBP per annum.¹⁵

Therefore, strategies that permit the early identification of biofilm formation, or onset of the CCT, would provide substantial benefits to both patients and healthcare providers by permitting early and more effective intervention. Unfortunately, clinicians presently do not have access to the tools with which to adequately address this challenge. The visual examination of wounds in general cannot provide evidence of biofilm formation, or unambiguous diagnosis of the onset of clinically relevant infection, unless this process is sufficiently advanced and extensive and mature biofilms are already established (which means treatment and healing has already been considerably undermined). In addition, the tools and techniques available for the study and examination of biofilms at early stages have been developed for fundamental laboratory research, and are not suitable or feasible for adaptation to clinical use^{16,17,18}

In contrast, the direct detection of cytotoxins or other virulence factors in wounds may constitute a valuable clinical indicator of CCT or early biofilm formation, and enhance clinical decision making. Because many bacterial species (including those most commonly associated with wound infections) regulate virulence factor expression based on population density,^{19,20,21} the CCT and biofilm formation is likely to be marked by activation or increase in expression of virulence factors, such as cytotoxins, by predominant wound pathogens. Direct detection of virulence factors would have important advantages in this application over traditional methods for wound microbiology, which rely on cultivation and assessment of bacterial cell numbers. Virulence factor detection stands to clearly link diagnosis to clinically relevant events in the pathogenesis of wound infection, whereas enumeration of cells in a wound is already considered a poor marker for the onset of wound infection, and provides no insight into biofilm formation.^{4,5} Detection of toxin expression should also permit the development of sensitive assays to allow intervention early in the transition to the CCT, before the significant tissue damage or extensive biofilm formation can occur.

Recently, we have demonstrated the potential for development of such diagnostic tests, and the “triggered release” of compounds in response to the onset of clinically relevant infection (CCT and biofilm formation), but not low-level background colonisation.^{22,23} This is accomplished through the use of synthetic lipid vesicles loaded with fluorescent self-quenching dye, which are sensitive to the key population-density regulated cytotoxic virulence factors produced by major

wound pathogens^{19,24,25}. These vesicles were found to be stable under conditions reflective of the un-infected wound environment,²⁶ but production of cytotoxins by bacterial pathogens results in lysis and dye release, which may be used to provide a clear signal that the CCT has been reached, and clinically relevant infection has been initiated.^{19,24} The natural evolution of this technology is now the incorporation of vesicles directly within wound dressings, to produce “intelligent”, infection-responsive, theranostic devices that can notify clinicians of the need to intervene directly at point of care.^{22,24,25} In this report, we present the first description of the development of a prototype “intelligent” wound dressing, which generates a clearly visible color change when activated by contact with bacterial biofilms in various model systems. In conjunction we also report the development of a more representative and realistic *ex-vivo* model of wound infections for the initial evaluation of these novel dressings.

EXPERIMENTAL SECTION

Materials. Agarose, human serum albumin (Human Male AB), cholesterol, 10,12-tricosadiionic acid (TCDA), 5,6-carboxyfluorescein, sodium chloride, sodium hydroxide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphate buffer saline (PBS), hexamethyldisilazane (HMDS), paraformaldehyde and glutaraldehyde were purchased from Sigma Aldrich, U.K. Lipid vesicles were made of phospholipid, cholesterol and polydiacetylene polymer lipid (TCDA). Phospholipid 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was acquired from Avanti Polar Lipids. Two modified HEPES buffers (with and without 50 mmol dm⁻³ 5, 6-carboxyfluorescein), containing NaCl and NaOH, were prepared in de-ionized water (18.2 MΩ cm, Millipore) and sterilized. Luria Broth (LB), Tryptic Soy Broth (TSB), Brain Heart Infusion (BHI) agar were supplied from Fluka and Fisher Scientific. Broths were dissolved in de-ionized water, sterilized and used in liquid bacteria culture. BHI agar plates were likewise prepared in 90 mm diameter petri-dishes and used as a nutrient agar support for the biofilm growth. The dressing mould was made of a square plastic plate with an array of short cylindrical studs to create an embossed hydrogel dressing. Biofilms were formed on 19 mm diameter porous polycarbonate membranes with the average pore diameter of 200 nm (Whatman). Human serum was mixed with freshly withdrawn blood (5%), aliquot into 500 µL small tubes and stored at -20 °C until further use.

Vesicle preparation. DPPC, cholesterol and TCDA were first individually prepared in

chloroform to a standard concentration of 100 mmol dm⁻³. Then DPPC, cholesterol and TCDA (660, 240 and 300 µL respectively) were mixed and dried in a glass vial with a flow of nitrogen. Resulting lipid film was further dehydrated in a vacuum chamber at 10⁻³ mbar for an hour to eliminate residual chloroform. Rehydration of lipid film was carried out by adding 10 mL of HEPES buffer (with 50 mmol dm⁻³ carboxyfluorescein) and the lipid mixture was heated at 70 °C for 10 minutes before performing three freeze-thaw cycles. Finally the lipid mixture was extruded three times through a polycarbonate membrane (25 mm dia. 200 nm pore size), using a Liposofast vesicle extruder (Avestin) heated at 55 °C. Non-encapsulated carboxyfluoresceins were then removed by filtering through NAP-25 gel columns (GE Healthcare) pre-washed in HEPES buffer. After a storage of purified vesicles at 4 °C for a week, UV polymerization of vesicles was carried out in quartz vials using a commercial flood exposure UV source (Hamamatsu, Japan) equipped with UV light (254 nm) with a total dose of 90 mW cm⁻². Vesicles were then stored at 4 °C and used within a month.

Bacterial culture. Four bacteria species comprising ten strains in total were studied and listed in table 1. They were *Escherichia coli* strain (DH5α), *Staphylococcus aureus* strains (MSSA476, RN4282, USA300, Agr⁺, Agr⁻), *Pseudomonas aeruginosa* strain (PAO1) and *Enterococcus faecalis* strains (E43, E57, E68). DH5α and Agr⁻ were attenuated strains from pathogenic origin and used as the controls in this study. The remaining strains of *S. aureus*, *P. aeruginosa* and *E. faecalis* were pathogenic, known to be associated with various virulence factors. *E. coli*, *E. faecalis* and *P. aeruginosa* strains were cultured in LB while *S. aureus* strains were cultured in TSB. Bacteria stocks stored at -80 °C were plated on LB and TS agar, following the incubation at 37 °C for 18 h. Bacteria from a single colony were enriched in LB and TSB at 37 °C in a shaker incubator (120 rpm) for 18 hours. Cultured bacteria were either used to prepare the supernatant or use for the biofilm growth. To prepare supernatant, the 18 h culture was first centrifuged (5000 rpm for 15 minutes) and then filter-sterilized (0.22 µm filters) before storage at 4 °C. For biofilm preparation, the culture was diluted in PBS buffer and adjusted to 3×10⁶ CFU/mL (colony forming unit per milliliter). For the study of dressing response to variable CFU in biofilm, initial inoculum contained approximately 30 to 3000 bacterial cells.

Preparation of prototype dressing. HEPES buffer (without carboxyfluorescein) containing 0.7, 1 and 2% agarose (%W/V) were heated in a microwave oven at 540 W for 3 minutes and

adjusted to 50 °C in an oven. Approximately 25 mL of agarose was required to make a square hydrogel dressing with a dimension of 95×95×2 mm³ (W×L×H). 1% or 2% agarose solution was then poured onto a mould, allowed to set at room temperature for a few minutes before further setting at 4 °C for about an hour. Removal from the mould results a thin hydrogel slab engraved with equally spaced, 144 cylindrical wells each bearing a uniform diameter and a depth of 4 mm and 1.5 mm respectively. Fillings in the pockets were a mixture of vesicles and 0.7% agarose (1:2 in volume). Vesicles and 0.7% agarose were pre-heated to 37 °C, then mixed and kept at 40 °C in a glass bottle inside a heated water bath. The mixture was then promptly pipetted into the wells of agarose dressing (approximately 35 µL/well) and allowed to set at room temperature before the storage at 4 °C until further use. Above mentioned production of prototype dressings were aseptically prepared inside a class II microbiological safety cabinet using sterilized apparatus.

Table 1 List of bacteria studied in the development of wound biofilm models

Species	Strains	Virulence activity	Reference
<i>Escherichia coli</i>	DH5α	Control	25,26
<i>Staphylococcus aureus</i>	MSSA475, RN4282, USA300, Agr+, Agr-	Pathogenic (except Agr-)	19,22,25
<i>Pseudomonas aeruginosa</i>	PAO1	Pathogenic	22,25,26
<i>Enterococcus faecalis</i>	E43, E57, E68	Pathogenic	27

***In-vitro* wound biofilm model (colony biofilm).** The wound model developed was a modified form of colony biofilm on an agar surface with additional materials which may facilitate the model to resemble the wound biofilms.²⁸ In biofilm preparation, a polycarbonate membrane was first sterilized in 70% ethanol for 15 minutes, dried and placed on BHI agar surface (with a shiny side facing up) under the flow hood. For the surface preconditioning of membrane, 30 µL of human serum containing 5% blood was evenly spread on membrane and allowed until the surface is dried. Fifty microliters of diluted bacteria in PBS buffer was then pipetted onto the center of PC membrane and the agar plate was incubated at 37 °C for 24, 48 and 72 hours. After desired periods of incubation, the membrane with a biofilm was removed and placed onto hydrogel dressing (facing down) inside the NUNC-12 well plate for fluorescence response study.

Dynamic biofilm model on The Modified Robbins Device (MRD). A chemostat system

was used to generate a continuous bacterial pure culture of *S. aureus* RN4282 in order to provide inoculum for use in the Modified Robbins Device (MRD) as described by Jass and colleagues²⁹ with some modifications. The culture was grown in a 500 mL double wall chemostat vessel (Tyler Research Corporation, Canada) with constant stirring at 300 rpm at controlled temperature ($37\text{ }^{\circ}\text{C} \pm 0.2$). A 10 ml sample of an overnight culture with 10^8 CFU/mL was used to inoculate 500 ml of sterile fresh TSB. Following incubation, the stirred chemostat was operated as a batch culture for four hours to allow the establishment of the culture. After, the medium volume inside the vessel was kept at 500 mL and perfused sterile TSB at a dilution rate of 0.1 h^{-1} . The chemostat was run over 48 hours for the culture to achieve the steady-state, meaning a constant population size of 4.5×10^7 CFU/mL. A stainless steel Robbins Device (Tyler Research Corporation, Canada) was used to establish a biofilm. The device provides quantifiable samples of biofilm that can be monitored over time. It consists of hollow rectangular stainless steel tubes with 12 evenly spaced sampling ports. Each port allowed the insertion of a sampling stud that contained a stainless steel disk that lay flush to the inner surface of the lumen being subjected to the flow media and bacteria. Each stud was covered with a PC membrane where the bacterial cells attach and form the biofilm. The MRD was fitted into a thermal liquid regulated container (Tyler Research Corporation, Canada) in order to maintain the internal lumen temperature at $37\text{ }^{\circ}\text{C}$. The culture growing under steady-state was pumped through the MRD over 24 hours, via the effluent tubing (flow rate = $250\text{ }\mu\text{L}/\text{min}$) of the chemostat and biofilms were allowed to develop. Over this period the culture feed was replaced by fresh half strength media supplemented with 0.1 % D-(+)-glucose (TSBg or LBG) for another 24 hours. At this time point, biofilms had been growing for 48 hours and biofilms started to be collected for analysis. The inlet and outlet tubing of the Robbins Device were clamped and four random studs were removed for analysis. Studs were removed at: pre-determined time intervals 24, 48 and 72 h.

Characterizations of biofilm models. Developed biofilms were characterized by crystal violet (CV) stain assay, standard plating for colony count, confocal laser scanning microscopy (CLSM) with Backlight Live / dead stain, and scanning electron microscopy (SEM). To determine the total biomass grown on membrane, the colony biofilm was stained with 0.1% CV for 20 minutes, before rinsing in PBS and drying at room temperature for an hour. It is worth mentioning that CV staining procedure of biofilms was slightly modified to meet the requirement of this study. Model biofilms are not rinsed in buffer prior to staining, as not only biofilm matrix

but also embedded microcolonies contribute to the virulence activities in infected wounds *in vivo*. Each biofilm was thoroughly re-suspended in 1 mL of 95% ethanol followed by 10 fold dilution OD measurement in a 96 well microliter plate. OD was measured using SPECTROStar Omega microplate reader from BMG Labtech (U.K.) at 600 nm. Three repeats per biofilm (200 μ L per well) were used to access the CV stained biofilm.

For quantitative analysis, each biofilm was thoroughly stripped off from the membrane in 1 mL PBS buffer by vigorous vortexing, before serially dilute in PBS buffer and plated on agar for standard colony count. For qualitative analysis, each biofilm was stained with CYTO live/dead stain and imaged using CLSM (Zeiss LSM510META). Live/dead stain was prepared according the manufacturer's instructions and each biofilm was stained for 15 minutes, followed by rinsing in PBS buffer. CLSM of biofilm provides the viable condition of each biofilm while SEM reveals the structural matrices of biofilm in detail. SEM sample preparation of biological samples is described in literature.³⁰ Briefly, each biofilm was fixed in a solution mixture of 3% paraformaldehyde and 1.5% glutaraldehyde in water for an hour at room temperature. Then biofilm dehydration was taken place in 50, 70, 80, 90 and 100% ethanol for 10 minutes each. Finally the biofilm was dried in HMDS for 30 seconds followed by 10 minutes air dry at room temperature. After mounting on an aluminium plate, a few nanometer thin conductive gold film was coated on biofilm by sputter coating before imaging with SEM (JEOL SEM6480LV).

***Ex-vivo* porcine skin burn wound model.** Freshly produced organic porcine skins were acquired from a local organic farm and used to create contact, burn wounds for *ex-vivo* wound biofilm study. Porcine skins were prepared aseptically under the flow cabinet using sterilized appliances. First the skin was thoroughly washed in deionized water, followed by disinfecting using 70% ethanol for 20 minutes before it was allowed to dry and diced into square pieces (approx. 3.5 \times 3.5 cm²). Sterilized brass blocks with contact area of 1.9 \times 1.9 cm² were heated to 170 °C and scald burns were created on each porcine skin for a skin contact of 20 seconds. The wounds were considered as second degree partial thickness burns bearing blisters. The edge of porcine skin was disinfected using standard betadine iodine solution in water (50 % V/V) before inoculation the wound with 50 μ L of bacteria (approx. 10⁵ CFU/mL) in respective media. The wounds were then incubated at 37 °C for 24 h.

Prototype dressing characterizations. The effect of virulence factors, associated with bacterial supernatant, on the fluorescence response of prototype dressings were tested in NUNC-12 well plate. Fluorescence released from each dressing was measured using the same Omega microplate reader by excitation and emission wavelengths of 485 ± 12 and 520 nm respectively. Finished dressing was cut into squares of $1.6 \times 1.6 \text{ cm}^2$, with each square contains 4 vesicle wells and each dressing was placed inside each well. The dressings were inoculated with 500 μL of supernatant per well (two dressings for each supernatant for average and standard deviation) and the plate was stored inside the incubator at 37°C . The fluorescence release from each dressing in response to supernatants at 0 and 5 hour time points were measured using a well scan mode (15×15 array of point measurements per dressing per well) and the average signal release per well was analyzed. To assess the dressing on the biofilm, the same dressing was used and the same experimental procedure was performed. Each biofilm was placed facing down on each square dressing in well plate. Using well scan mode, the average fluorescent signal released by each dressing from 0 to 7 hour time points were measured and analyzed.

RESULTS AND DISCUSSION

Clinical assessment of prevalent burn wound pathogens. *S. aureus*, *P. aeruginosa* and *E. faecalis* are commonly reported as the most prevalent pathogens causing wound-related complications in various types of wounds.³¹ To confirm their prevalence in burn wounds, we undertook our own independent study based on clinical records for burn wounds at Queen Victoria Hospital (QVH) and Bristol Royal Hospital for Children (BRHC). Data from QVH ($n = 55$) showed that almost three quarters of wounds surveyed were colonized by pathogenic bacterial species (figure 1A). Among these *S. aureus* was found to be by far the most prevalent pathogen recovered from colonized wounds and present in $\sim 71\%$ of wounds considered “at risk” of infection. The second and third most commonly encountered pathogens were *P. aeruginosa* and *E. faecalis*, which were present in $\sim 17\%$ and $\sim 11\%$ of colonised wounds respectively (figure 1B). A comparable profile of wound colonization was observed at BRHC for both adult and pediatric burns ($n = 25$).³² Collectively, this survey indicated that over 80% of wounds colonized by bacterial pathogens contained one or more representatives of this dominant *Staphylococcus*-*Pseudomonas*-*Enterococcus* group (SPE-group; figure 1B). Therefore, the development of strategies aimed at detecting wound infections caused by members of this dominant SPE-group

of pathogens became a primary consideration when designing our prototype infection-detecting intelligent wound dressing.

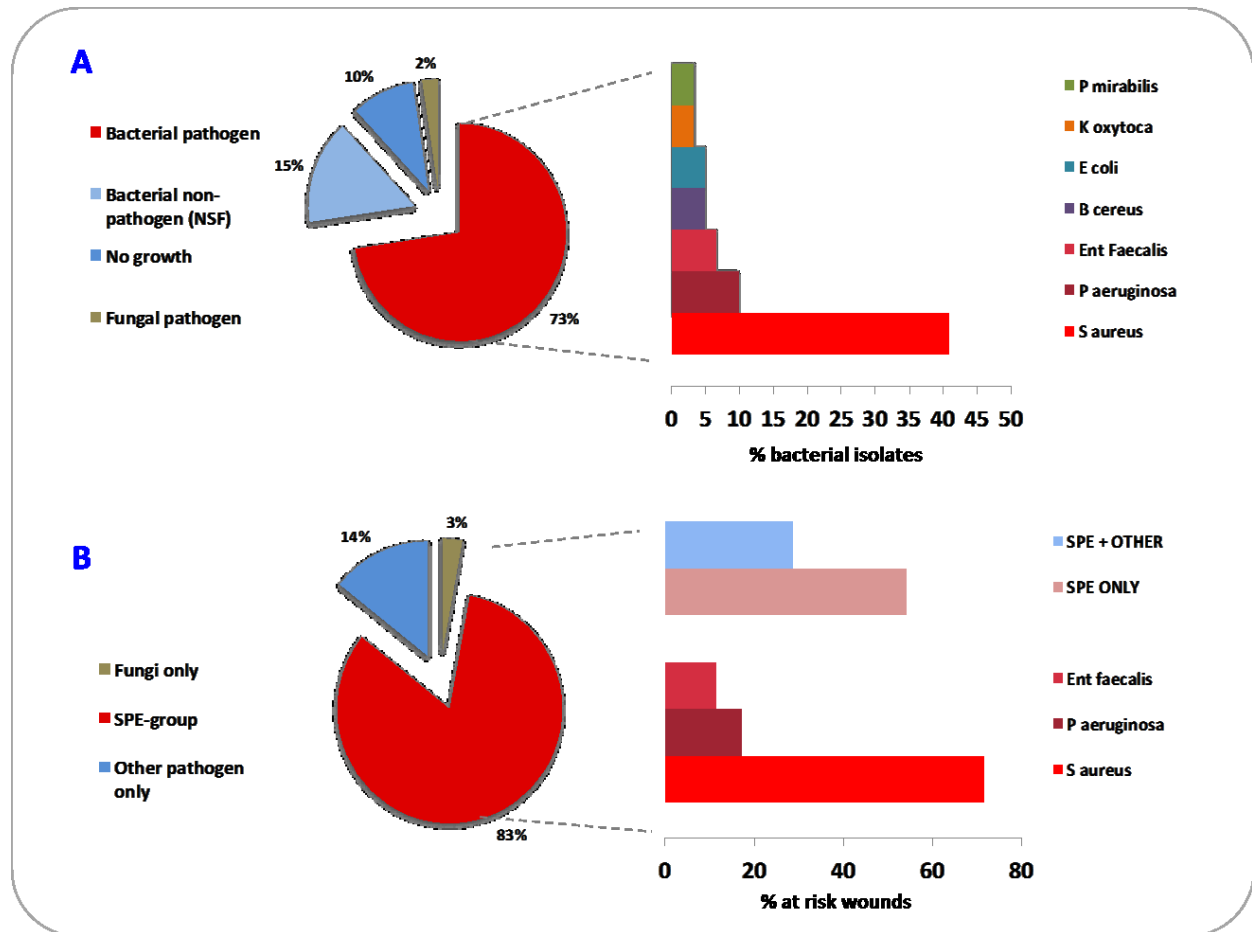


Figure 1 Proportion of burn wounds colonized by pathogenic microbes, and incidence of specific pathogens. (A) Proportion of burn wounds colonized by bacterial or fungal pathogens and considered “at risk” of infection. Associated bar chart shows breakdown of bacterial pathogens identified in “at risk” wounds as a proportion of total isolates (multiple isolates per wound). (B) Proportion of “at risk” wounds colonized by dominant SPE-group pathogens comprised of *S. aureus*, *P. aeruginosa*, and *E. faecalis*. Each wound was considered SPE positive if any SPE-group member was isolated, regardless of other species present. Associated bar chart provides detailed breakdown of at risk wounds according to presence of SPE-group bacteria and other pathogens, as a proportion of all examined wounds colonized by bacterial pathogens. In total 83% of wounds colonized by bacterial pathogens and at risk of infection were positive for SPE-group pathogens. Data derived from records of patients treated for burns at Queen Victoria Hospital (n=55).

Design evaluation of the prototype dressing. The mechanism by which the fluorescent signal propagates from lysed vesicles is an important design consideration of the prototype dressing. The dye used in this study is 5, 6-carboxyfluorescein, which forms dimers above

self-quenching concentration.³³ At the concentration achieved inside vesicles the dye is non-fluorescent, but when released and diluted in the surrounding aqueous environment self-quenching is inhibited and fluorescence activated. The schematic representation of the dressing production is depicted in figure 2A. Initial attempts produced a hydrogel film which was made of a mixture of vesicles with agarose. Subsequent improvements were made by patterning vesicles in the regular array of wells, which upon release provides the immediate dilution of fluorescence into the surrounding agarose (figure 2B). The dressing activation was demonstrated using the detergent Triton to lyse vesicles, and demonstrated the “activation” of fluorescence and the clear and uniform signal generated across the entire dressing (figure 2C).

Effects of agarose content in dressing stability and response was also evaluated. When using 1% agarose, fluorescence activation using Triton was relatively quicker as the hydrogel contained more water but the rigidity of dressing was affected by high water content. On the other hand, 3% agarose increased the stiffness of dressing with slower and weaker fluorescence activation. The optimum agarose content was 2%, as it supports both dressing stability and reasonable dye diffusion. It was also noted that the majority of dye was just retained within the hydrogel film, which may help minimize the fluorescence cross-over to the wound in practice, and serve to retain signal strength over a longer period of time. The current design also permits the diffusion of virulence factors from the wound bed into the dressing, which is of crucial importance for activation of vesicles. In relatively larger wounds such as burns, it is almost impossible to locate the contaminated local area(s) where pathogens begin to colonize and act as foci of infection, before it reaches the critical colonization threshold (CCT) and spreads all over the wound. However, a more detailed knowledge of the wound microbial landscape may be highly beneficial in treatment and wound management, by guiding clinicians to areas of infection, and enabling them to prioritize a localized and sustained treatment of infected regions, particularly during surgical debridement. In this regard the wound mapping of infection could be possible with this dressing design.

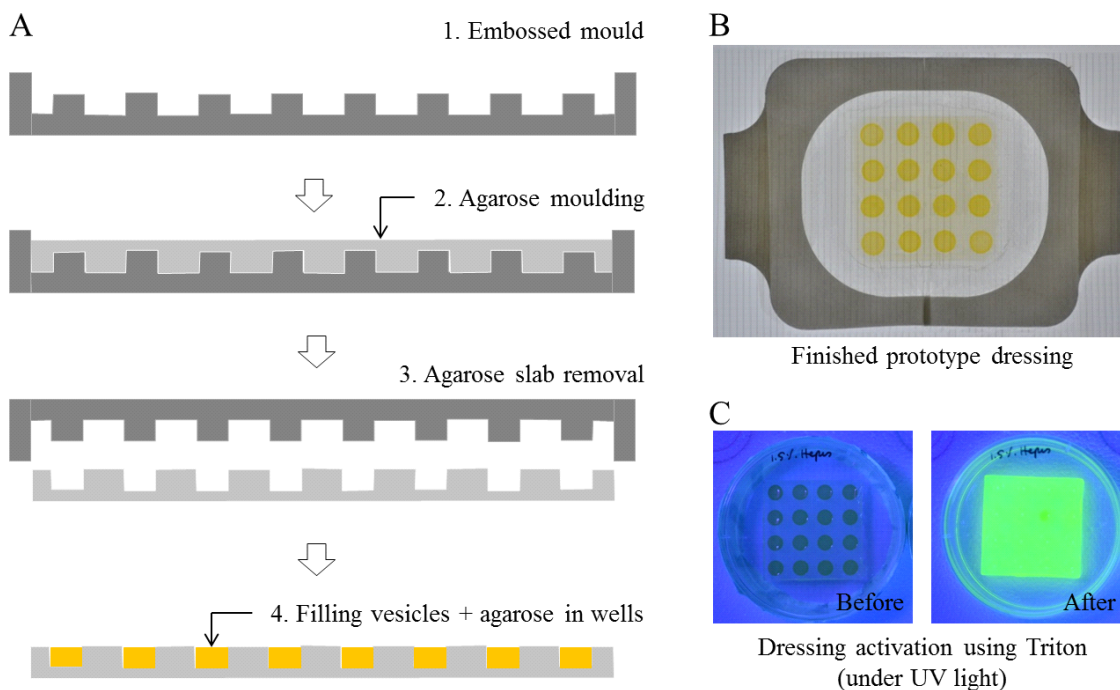


Figure 2 Design and development of the intelligent hydrogel wound dressing; A) schematics of the dressing depicting the stages of production, B) a finished prototype dressing on a transparent polypropylene film, and C) the same dressing before and after the activation using Triton to be seen under UV light

Analysis of colony biofilm models. A wound under a protective dressing can be considered as a closed environment. Biofilms typically form on the surface of the wound with nutrients, derived from damaged tissues and exudate, obtained from the underlying wound bed. An agar matrix is supplemented with chopped meat-based nutrients (such as Brain Heart Infusion, BHI), as well as blood plasma and erythrocytes, is therefore able to replicate conditions similar to those encountered in the wound bed and provide a basic wound biofilm model. In our model, BHI agar was used as a source of nutrients and a biofilms were grown on a nano-porous polycarbonate (PC) membrane laid on the agar surface.

Growth of biofilm on a PC membrane provides several advantages.²⁸ Nano-pores prevent bacteria crossing the membrane while ensuring the bacteria to access nutrients from underlying agar, and allowing them to form a biofilm on the membrane surface. Additionally, the wound biofilm model provides a simple method of preparation, a wide choice of agar nutrients with a control of growth conditions. Importantly, it permits easy handling of biofilms and facilitates their recovery from wound models from agar surfaces for further processing and analysis. The

membrane supporting a biofilm can be transported easily for various characterizations, such as crystal violet staining to estimate levels of biomass, recovery of viable cells for plating and enumeration, and confocal and scanning electron microscopic imaging of biofilm. Schematic representation of a colony biofilm developed on an agar surface is depicted in figure 3A.

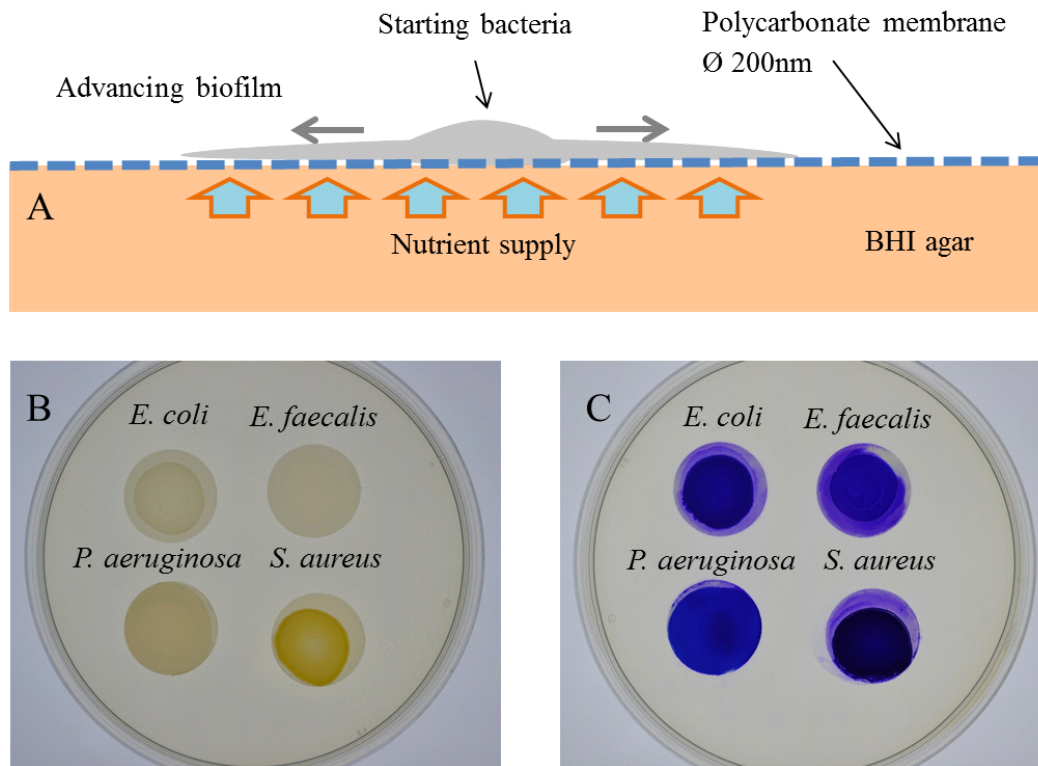


Figure 3 Colony biofilm model development; A) the schematic depiction of the colony biofilm model, and the 48 h growth of colony biofilm on nano-porous polycarbonate membrane before (B) and after (C) crystal violet staining

Growth of biofilms was optimized by varying initial inoculum of bacteria, serum pre-treatment of the membrane, and incubation time and temperature. Regarding incubation time there was no difference in biofilm formation for all species except *P. aeruginosa* biofilm in which growth expanded beyond the edge of membrane after 48 h of incubation. This was due to a motile nature of gram-negative *Pseudomonas* (swarming) in contrast to the non-motile *S. aureus* and *E. faecalis*, which showed slow radial growth of biofilm. Colony biofilms formed by *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecalis*, grown at 37 °C for 24 h are shown in figure 3B, followed by CV staining (figure 3C). It was clearly seen that *P. aeruginosa* produced a relatively thick and expanding biofilm. The biofilm, when examined under UV light, emitted auto-fluorescence,

which otherwise were not observed in planktonic growth. These biofilm associated green pigments, called pyocyanin are known to be secreted by *P. aeruginosa* and they exhibit redox properties and help the biofilm to mature although virulence activities of pyocyanin are not well known.³⁴

SEM characterization of model biofilms after 48 h contained bacteria interlinked within the networks of secreted extracellular polymeric substances (EPS), though physical appearance differed between species (figure 4). Biofilms formed by the Gram-negative, motile *E. coli* and *P. aeruginosa* appeared to be more extensive than (figure 4A and 4B), biofilms generated by Gram-positive *S. aureus* and *E. faecalis* in which cells appeared more densely packed in general (figure 4C and 4D). Additional SEM images at lower magnification were presented in supporting information session (Figure S1). Both SEM and CV staining provided the evidences of successful biofilm formation on PC membranes. Biofilms matured for 24, 48 and 72 h periods were studied and their respective biomasses were estimated by CV staining (figure 5B). *P. aeruginosa* biofilm was observed increasingly growing up to 72 h and the same was confirmed by SEM and visual observation. *P. aeruginosa* is considered a particularly adept at forming biofilms relative to many other bacterial species³⁵ and its colonization in lungs of patients with cystic fibrosis (CF) is typically linked to the formation of biofilms.³⁶ In our model, a constant supply of surface nutrients facilitates the three dimensional growth of biofilms on the membrane, which might be enhanced by the surface associated swarming motility displayed by *P. aeruginosa*. It is likely that swarming facilitates the migration of cells over the membrane and agar surface, prior to initiation of biofilm formation, increasing the area over which biofilms form and leading subsequently to greater levels of overall biofilm formation. There was no noticeable decline of *P. aeruginosa* population (CFU per biofilm) observed up to 72 h growth. Importantly live/dead stain assay of *P. aeruginosa* (24 h) biofilm examined using CLSM provided the evidence of a healthy biofilm (figure S2 in supporting information). Therefore it may consider that *P. aeruginosa* biofilm constantly expands with increasing synthesis of biofilm matrix (evidenced by CV staining in figure 5B), while the population density of healthy, otherwise EPS producing bacteria seem to maintain as the biofilm matures.

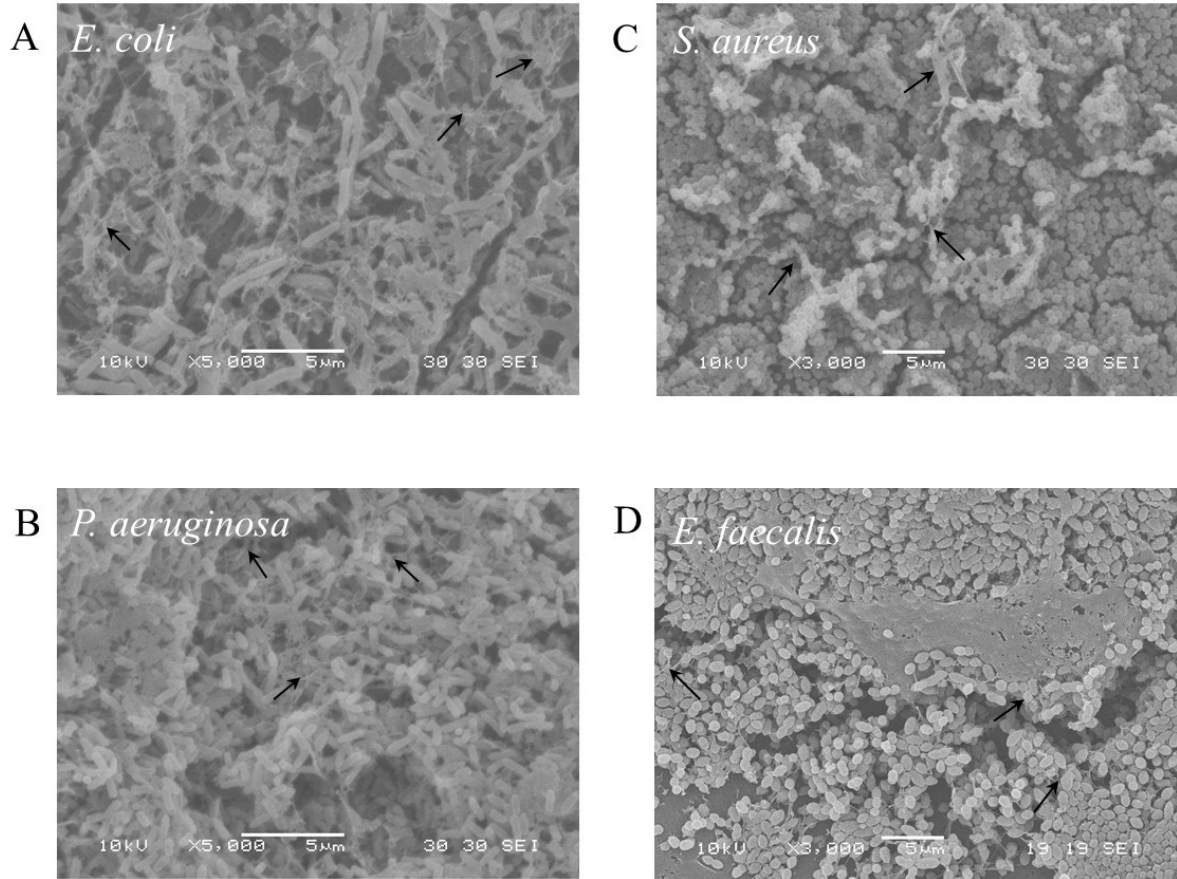


Figure 4 SEM images of colony biofilms belong to A) *E. coli*, B) *P. aeruginosa*, C) *S. aureus* and D) *E. faecalis*. *E. coli* and *P. aeruginosa* formed a complex, extensive biofilms while *S. aureus* and *E. faecalis* comprised a relatively densely packed cells in biofilms. Arrows indicates the EPS networks (scale bar - 5 μ m).

In contrast *S. aureus* and *E. faecalis* biofilms reached a steady state of growth in 48 h and remained unchanged up to 72 h (figure 5B). Analysis of these biofilms showed a gradual decrease in CFU per biofilm from 48h to 72 h growth, especially for *S. aureus* (figure 5A). As the thickness of the biofilm increases, cells embedded within the biofilm are likely to be deprived of nutrients and oxygen, and in mature biofilms many cells have been show to enter a metabolically inactive, dormant state, and may be viable but non-cultivable.^{37,38} This phenomenon can potentially lead to a discrepancy between plate counts of bacterial cells and the actual number of viable cells in biofilm. Notably, quantification of *E. coli* biofilms through CV staining of total biomass, shows similar discrepancies, with CV staining indicated *E. coli* to exhibit the greatest level of biofilm formation at all-time points in contrast to CFU based evaluation using plate counts.

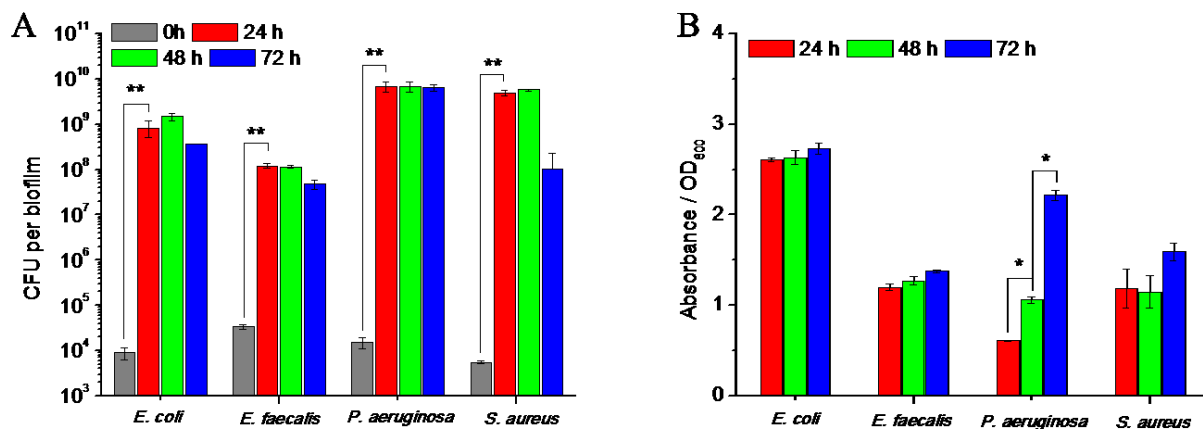


Figure 5 Characterization of *E. coli* and SPE group colony biofilm models grown for 24, 48 and 72 h; (A) microbial population of the biofilm bacteria with the biofilm forming time derived from standard colony count, and (B) biomass estimation of the colony biofilms using CV assay against growth duration (* $p < 0.005$ and ** $p < 0.05$)

Dressing response to supernatants and colony biofilm model. Colorimetric assessments of the prototype dressing were carried out using bacterial supernatants and colony biofilm models on PC membrane. Bacterial supernatant was a filtered LB/TSB media of planktonic bacteria cultured at 37 °C for 18 h, which contains metabolic by-products of bacteria comprising various forms of exotoxins, enterotoxins, secreted proteins, enzymes and polypeptides. Dressing response test using the pathogenic bacterial supernatant permits a rough estimation of the impact on vesicles and the concentration of virulence factors generated by species of interest under study conditions. The dressing response to the supernatant is useful in parallel with study of the dressing response to colony biofilms. The test results were presented in figure 6. *S. aureus* supernatants, within 5 h of inoculation, activated the dressing completely (figure 6A), as it reached the same level of fluorescence as the Triton (positive control). Interestingly, a slightly weaker response of the dressing was observed when tested with the colony wound biofilm models composed of the same strains of *S. aureus* (figure 6C). In general the longer the biofilm growth, the lesser response of the dressing was noticed (figure S3 in supporting information). Nevertheless there was almost no difference in fluorescence triggered by supernatant and biofilms when observed by eye under UV light. Selectivity of the dressing was clearly demonstrated by the lack of fluorescent response to negative controls: i.e. HEPES and non-pathogenic *E. coli*, compared to a clear and strong activation of fluorescence by pathogenic

strains of *S. aureus* and *P. aeruginosa*.

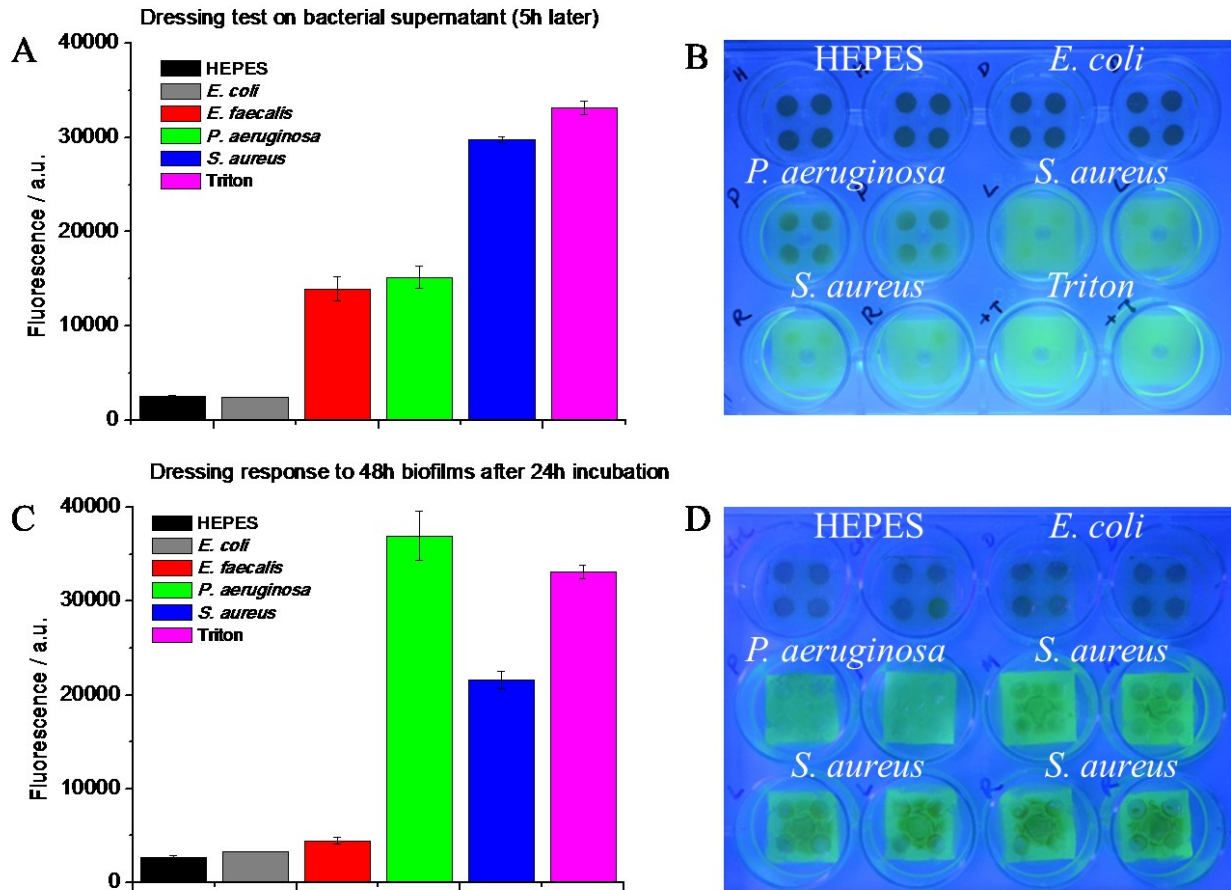


Figure 6 Prototype dressing responses to bacterial supernatants and colony wound biofilm models; fluorescence triggered release by the supernatants (A) and colony biofilm models (C) of control and SPE pathogenic bacteria. Photographs taken under UV light of the colorimetric response of the dressings 5 hours after inoculation with supernatants (B) and same dressings response to 48 h colony biofilms after 24 h of incubation (D)

In the supernatant test, the fluorescence response of the dressing to *P. aeruginosa*, however was relatively slow but gradual dilutions of fluorescence from the vesicle wells of the dressing was noticed. However, when tested with biofilms, *P. aeruginosa* showed higher responses which are most likely attributed to virulence factor production associated with biofilm formation and quorum sensing activated gene expression (figure 6C). Plating and colony count data revealed that CFU per *P. aeruginosa* biofilm remained almost the same regardless of the growth duration (figure 5A). CLSM study of biofilms also confirmed that *P. aeruginosa* produced a relatively

thick biofilm with the majority of cells retaining viability (figure S2 in supporting information). A time course analysis of *in-vitro* *P. aeruginosa* biofilms previously showed increased secretion of all virulence factors as biofilms matured up to day 4.^{39,40} A significant dressing response therefore was believed to be triggered by increased production of virulence factors associated with actively growing biofilms. In contrast to *S. aureus*, the response elicited by *P. aeruginosa* biofilms was correlated with the age of biofilms, with the strongest response triggered by the oldest biofilms (figure S2 in supporting information).

E. faecalis supernatant induced a relatively weak dressing response, similar to *P. aeruginosa* supernatant, but also only a low level response from *E. faecalis* colony biofilms (data not shown). Cell membrane lysing toxin produced by *E. faecalis* is a two component cytolysin which has established lytic activity against both bacterial and eukaryotic cells.^{41,42} Although the production of this toxin is regulated by a quorum sensing auto-induction mechanism, *E. faecalis* also appears to regulate toxin production in response to target cells, and represses toxin production in their absence even when population density reaches threshold levels.⁴³ In the present study, the lack of target cells available in planktonic culture or biofilm models of *E. faecalis*, is likely to have reduced cytolysin expression leading to negligible levels in culture supernatant. This in turn would reduce the capacity of *E. faecalis* supernatants to lyse lipid vesicles and generate a clear response from dressing prototypes. Although, PC membranes in biofilm models were pre-treated with serum and blood, appears inadequate to simulate target cells and facilitate cytolysin production sufficient to activate dressings

Colony biofilms initiated with varying initial inoculum densities of bacterial cells were also studied to quantify the lowest initial inoculum capable of activating dressings after 24h of the dressing. As expected, when the initial inoculum was low (less than hundreds of CFU), only individual isolated colonies were observed after 24h, which may be thought of as discrete biofilm structures in the context of the colony biofilm wound model. At these low levels of colony biofilm formation, there was no dressing response from *E. coli* and *S. aureus* (Agr-) regardless of CFU in each biofilm (figure 7A). Again *E. faecalis* failed to produce a response from dressings irrespective of CFU per biofilm. *P. aeruginosa* provided the highest but constant response triggered by 20 to 2000 CFU per biofilm, and may reflect a more rapid proliferation to higher cell numbers in *P. aeruginosa* compared to other pathogens; a higher sensitivity of

vesicles to *P. aeruginosa* virulence factors; the expression of high levels of virulence determinants in *P. aeruginosa* compared with other pathogens; or a combination of these factors. Pathogenic strains of *S. aureus* also showed increasing response with increasing CFU per colony biofilm (figure 7B). These results demonstrate the potential for dressings to detect early stages of biofilm formation at relatively low levels.

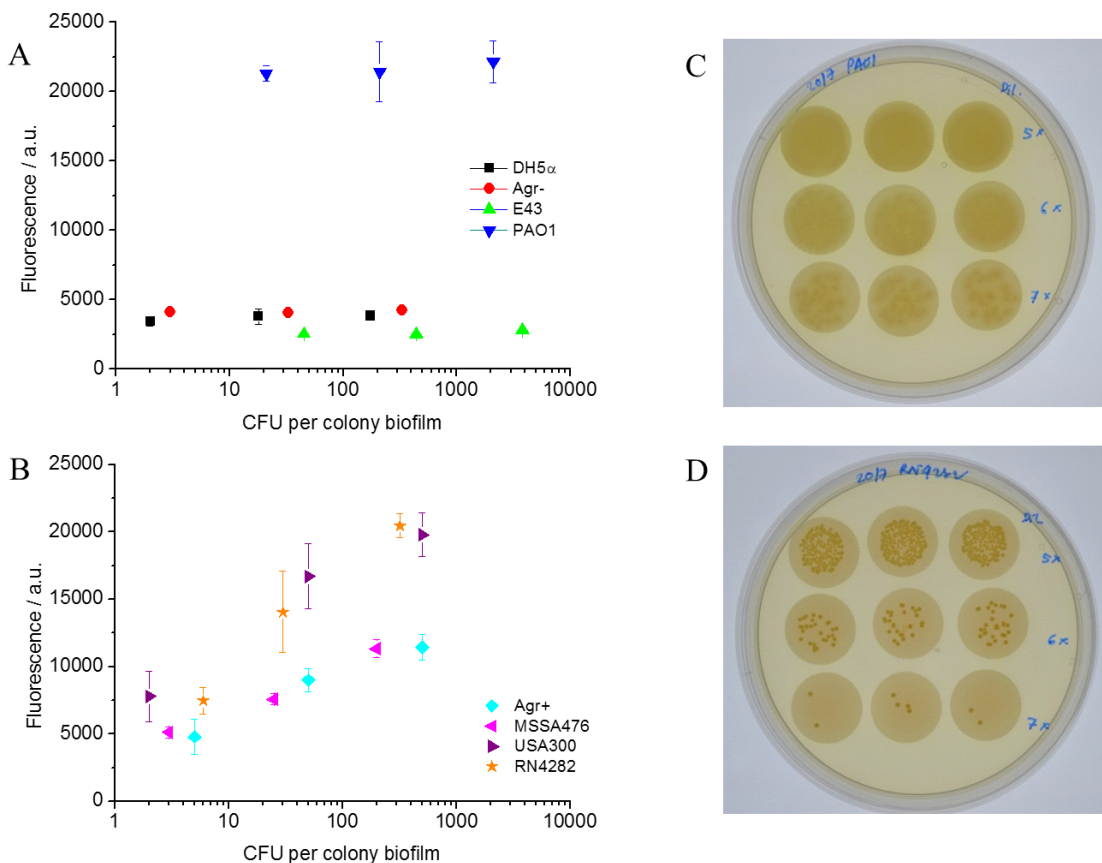


Figure 7 Quantification of minimum biofilm forming colonies required for the dressing response with (A) *P. aeruginosa* (PAO1), *E. faecalis* (E43) and controls *E. coli* (DH5a) and *S. aureus* (Agr-) and (B) pathogenic *S. aureus* strains (Agr+, MSSA476, USA300 and RN4282). PAO1 provided strongest fluorescence regardless of the CFU per biofilm from tens to thousands. *S. aureus* strains showed CFU dependent response. (C) and (D) are colony biofilms with varying initial inoculums of PAO1 and RN4282 respectively.

Colony vs. dynamic biofilm models. The structure and properties of biofilms formed by the same strain can vary depending on the physical environmental conditions. In particular shear forces generated by media flow are documented to result in mechanically stronger biofilms with altered phenotypes, compared to biofilms formed in low or no shear environments such as wounds.³⁷ Therefore, we also compared the response of dressings to *S. aureus* biofilms formed

under distinct conditions: i) the static, no shear environment of the rudimentary colony biofilm wound model ii) biofilms formed under media flow under more dynamic environmental conditions, generated using the Modified Robins Device (MRD). SEM and CLSM images of *S. aureus* showed increasing biofilm formation over time in the dynamic MDR system analogous to that observed in simple colony models (figure 8). Biofilms formed in simple colony models at 24 and 48 h generated a better dressing response than the MDR grown biofilms of the same age. A difference however was noticed in 72 h growth when MDR grown biofilms produced higher dressing response (figure 9). This could be the effect of biofilm maturation in dynamic model compared to a colony counterpart with the stationary growth conditions.

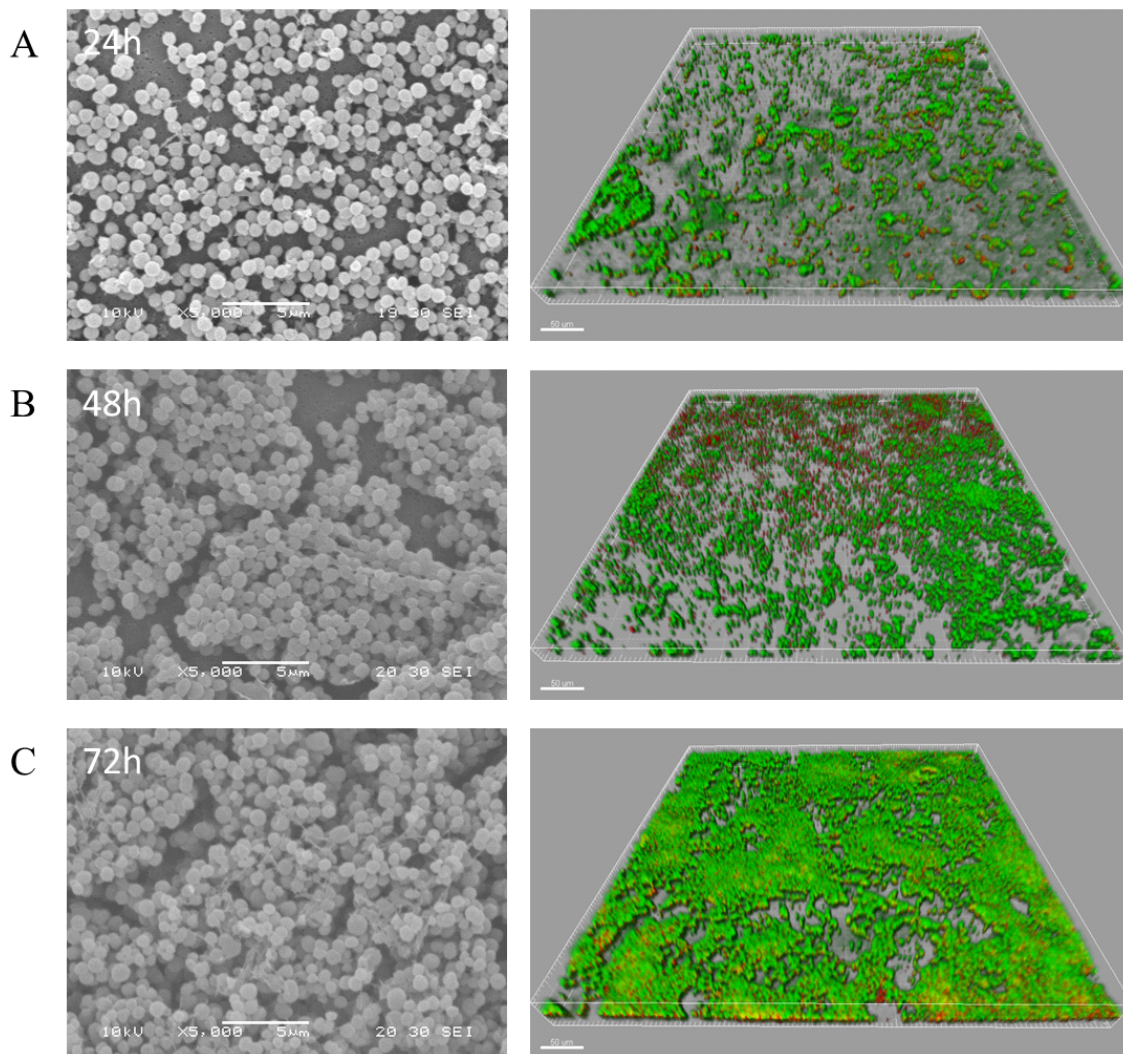


Figure 8 SEM and CLSM images of the dynamic biofilms of *S. aureus* (RN4282) grown by Robbins device for (A) 24 h, (B) 48 h and (C) 72 h. Expansion of EPS network and biomass increment was observed from biofilm forming times of 24 to 72 h (scale bars were 5 μm (SEM))

and 50 μm (CLSM)).

When growing on the Robbins device, the bacterium was supplied with a fresh flow of nutrients and given a selection on the growth modes of planktonic or sessile attachment. Except for 24 h growth showing a relatively sparse surface coverage by biofilms, the MDR appeared to display higher levels of biofilm formation between 48 and 72 h as assessed subjectively by CLSM visualisation (figure 8). As the secretion of virulence factors was closely related to the healthy growing condition of biofilm,^{39,40} this could explain the enhanced dressing response by 72 h dynamic biofilm over the colony biofilm of the same species (figure 9). However, the exact levels of biofilm formed per unit surface area in the distinct models were not calculated, and it also remains probable that the differences observed are predominantly a function of biofilm density achievable in each system.

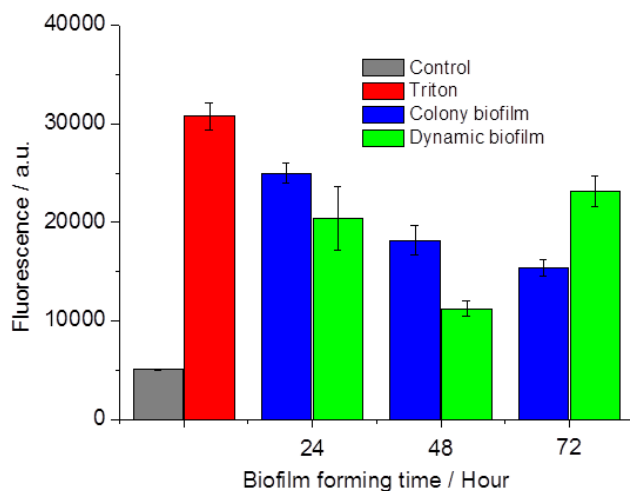


Figure 9 A comparison of the dressing fluorescence response between the colony and dynamic biofilms of *S. aureus* (RN4282). The vesicles lysing activity of colony biofilm was higher than dynamic biofilms with the exception for 72 h growth.

Porcine skin burn wound infection study. Though not without merit and utility, both the simple colony model and the more complex MRD provide only a partial simulation of wound biofilms, and both are subject to important limitations in this regard. Therefore, we also sought to develop a more representative and realistic model of wound biofilm formation with which to evaluate “intelligent” dressing designs. This led to development of an *ex-vivo* burn wound biofilm model using thermally damaged dermal tissues of porcine skin, and evaluation of dressing performance in this system. The second degree partial thickness burn on a porcine skin

section producing blisters is shown in figure 10A. Twenty four hours after inoculation with bacteria wound colonisation and presumptive biofilm formation was observed as a jelly like film on wound surface (figure 10B). When the dressings were applied, all infected wounds with pathogens, except for negative control of non-pathogenic *E. coli* clearly showed a fluorescence response from dressings (figure 10C). Although the picture was taken after 24 h post dressing placement, the significant response was seen in first 6 hours, especially in wounds infected with *P. aeruginosa* and *S. aureus*. *P. aeruginosa* secretes pyocyanin dye which fluoresces under UV light resulting in the blue color emission seen from the biofilms in figure 10C, not to be confused with the vesicle fluorescent dye activation and emission which is observed bright green / white. Most notably, this model also elicited a clear and overt response from *E. faecalis* in infected wounds which was not observed in previous tests using supernatant and simple colony biofilms. The presence of target cells such as endothelia and erythrocytes in porcine skin likely permits the target-dependant activation of cytolysin in this system, with sufficient expression induced to lyse vesicles in dressings. All in all SPE pathogens seemed to produce biofilms in porcine skin burn wounds which triggered the activation of the visible dressing fluorescence.

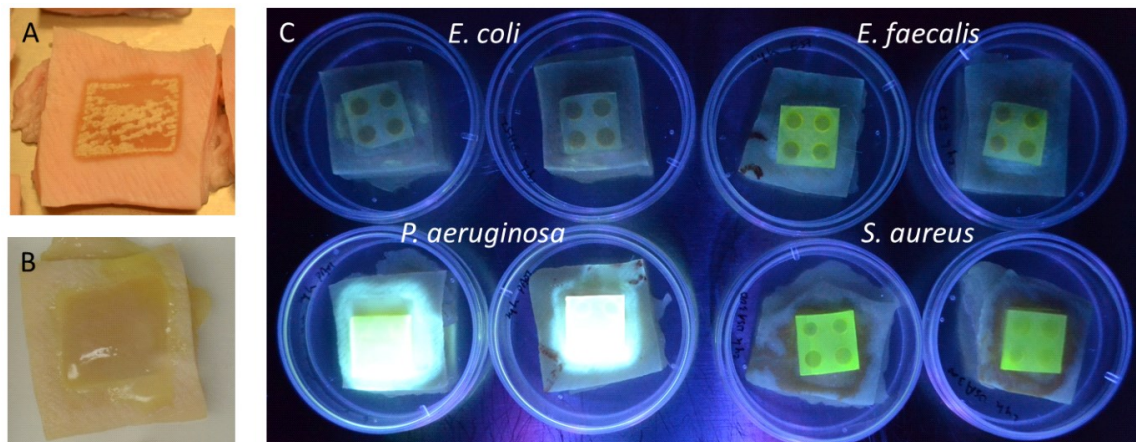


Figure 10 Porcine skin burn wound infection study showing (A) a second degree partial thickness burn wound with blisters, (B) the burn wound 24 h after infection with PAO1 and (C) the fluorescence response of prototype dressings on infected wounds of SPE pathogens 24 h later

CONCLUSION

A novel concept of detection of infection is presented, in which dye-carrying vesicles are contained in an intelligent hydrogel wound dressing. Dye release is triggered by virulence factors

of pathogenic wound biofilms. The dressing contains arrays of vesicle wells embedded inside the hydrated film of agarose. The colorimetric activation is triggered by virulence factors of pathogenic origin only, and not by non-pathogenic bacteria. A simple rudimentary colony wound biofilm model, which partly mimics *in vivo* wound biofilms is developed on a polycarbonate membrane and is suitable for initial high-throughput dressing evaluation. A clinically important and dominant group of wound pathogens (SPE-group) were tested and shown to trigger the fluorescence response of the dressing, when cultivated either as planktonic cells or as biofilms. *P. aeruginosa* and *S. aureus* biofilms generate a string fluorescence from dressings which is not seen in *E. faecalis* supernatant and biofilms formed under most model conditions, possibly due to the specific requirement of target cells in activation of *E. faecalis* cytolysins. This is supported by the good dressing response to *E. faecalis* when biofilms formed in the more representative *ex vivo* porcine skin burn wound models were tested. Overall, this study provides proof-of-concept for an advanced infection-detecting dressing for wound care, which could allow the targeted treatment of infections at the bedside and reduce the unnecessary use of antibiotics.

ASSOCIATED CONTENT

Supporting Information

Low magnification SEM images of 48 h colony biofilms (S1), CLSM image of PAO1 forming colony biofilm on nano-porous polycarbonate membrane after 24 h (S2) and the graph on fluorescence response of the dressings when tested with *P. aeruginosa* and *S. aureus* colony biofilms of 24, 48 and 72 h growth (S3). These materials are available free of charge via the Internet at <http://www.pubs.acs.org>.

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DECLARATION

The authors declare no conflict of interest.

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